# Identification of the second gonadotropin-releasing hormone in chicken hypothalamus: Evidence that gonadotropin secretion is probably controlled by two distinct gonadotropin-releasing hormones in avian species

(reproduction/neuropeptide)

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**ABSTRACT** A new peptide having gonadotropin-releasing activity distinct from the known luteinizing hormone-releasing hormones (LHRHs) has been identified in a chicken hypothalamic extract. The existence of [Gln⁸]LHRH in avian hypothalamus has been reported previously. The new molecular species of gonadotropin-releasing activity, named chicken gonadotropin-releasing hormone II (chicken GnRH-II), has been isolated recently in a yield of 7 $\mu$g, starting from 10,000 chicken hypothalami. Structural analyses have been performed on the peptide fragments derived from chymotryptic and thermolytic digests of chicken GnRH-II by amino acid analyses and terminal analyses. The full sequence of chicken GnRH-II has been determined to be: pGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH₂. A synthetic decapeptide with the above sequence was verified to be chromatographically identical to natural chicken GnRH-II. For further structural confirmation, chymotryptic and thermolytic peptides from synthetic and natural chicken GnRH-II also were identified. Thus, the structure of chicken GnRH-II has been definitely established. The gonadotropin-releasing potency of chicken GnRH-II was about 32% of that of mammalian LHRH and 8 times more potent than chicken LHRH, as estimated from the bioassay with rat anterior pituitary cells. Our results indicate that gonadotropin secretion is probably controlled by two distinct GnRHs, at least in avian species.

Since the structural determination of porcine hypothalamic luteinizing hormone (LH)-releasing hormone (LHRH) in 1971 (1), the concept has widely been acepted that gonadotropin secretion is controlled by a sole gonadotropin-releasing hormone (GnRH), such as mammalian LHRH. On the other hand, we have demonstrated that gonadotropin secretion is not controlled by LHRH under certain physiological and experimental conditions (2). However, there has been no structural evidence that two or more than two GnRHs coexist and function in any vertebrate.

The present paper represents the structural demonstration of the existence of two different GnRHs at least in avian species.

Recently, we (3, 4) and King and Millar (5–7) have independently isolated chicken LHRH and determined its structure as [Gln⁸]LHRH. We have purified chicken LHRH by monitoring its biopotency towards rat anterior pituitary cells. During the course of the purification, another gonadotropin-releasing activity was found in the chicken hypothalamic extract, which was well separated from that due to

chicken LHRH on ion-exchange chromatography.

The present paper describes the isolation and structural determination of the second GnRH in chicken hypothalamic extract (designated chicken GnRH-II).

Chicken GnRH-II has been purified by successive gel filtration, ion-exchange HPLC and reverse-phase HPLC. A pure peptide has been isolated in a yield of 7 $\mu$g (6 nmol), starting from 10,000 chicken hypothalamic fragments.

The amino acid composition of the peptide indicated a decapeptide structure consisting of Ser₁Glx₁Pro₁Gly₂Tyr₁-His₂Trp₂. By amino acid analyses and terminal analyses of enzymatic fragments derived from the purified decapeptide, the complete structure of chicken GnRH-II has been determined to be: pGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH₂.

The present paper also describes the gonadotropin-releasing activity of chicken GnRH-II compared with that of mammalian LHRH.

## MATERIALS AND METHODS

**Assay of Gonadotropin-Releasing Activity.** Gonadotropin-releasing activity was estimated on the rat anterior pituitary cell culture system as reported (3).

Anterior pituitaries were obtained from female Holzman rats (180- to 220-g body weight), agitated in trypsin solution (0.25%), and finally dispersed with 1% Viokase (GIBCO) solution. Cells were collected and washed three times with Dulbecco's modified Eagle's medium (DME medium) containing gentamycin (100 $\mu$g/ml), fungizone (25 $\mu$g/ml), 5% horse serum, 5% human cord serum, 2.5% bovine calf serum, 0.1% glutamine, and 1% nonessential amino acids (GIBCO). Cells were resuspended in an appropriate volume of the medium described above, and 0.2-ml portions (containing 5 × 10⁴ cells) were put into 96 multi-well tissue culture plates (Nunc, 1-67008). The culture plates were placed in an incubator at 37°C in a water-saturated atmosphere of 10% CO₂/90% air. The cells were precultured for 4–5 days. The culture medium was then replaced by 0.2 ml of DME medium containing samples, and cells were further incubated for 20 hr, an incubation period that is necessary for reliable measurement of follicle-stimulating hormone (FSH) released into the medium by the radioimmunoassay. Eighty- and forty-microliter aliquots of the resulting incubation medium were subjected to radioimmunoassay of FSH and LH, respectively. The biological potency of natural and synthetic chicken GnRH-II was determined and

Abbreviations: LH, luteinizing hormone; LHRH, LH-releasing hormone; GnRH, gonadotropin-releasing hormone; chicken GnRH-II, the second chicken GnRH; FSH, follicle-stimulating hormone.

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compared with that of mammalian LHRH.

**Isolation of Chicken GnRH-II.** Diced hypothalamic tissues from 10,000 chickens were boiled for 10 min in 30 liters of 1 M acetic acid and then extracted. The supernatant was passed through a membrane filter (UH1, Toyo Roshi, Japan) to remove salts and small molecules (<500 daltons), and then the content was lyophilized. The dried material was reconstituted with 1 M acetic acid and treated batchwise with SP-Sephadex C-25 (free form). Adsorbed material was first eluted with 2 M pyridine solution and then with 2 M pyridine acetate buffer (pH 5.0). Chicken LHRH was eluted in the 2 M pyridine fraction, while chicken GnRH-II emerged in the 2 M pyridine acetate (pH 5.0) fraction. The material, which was eluted in the 2 M pyridine acetate buffer, was then submitted to gel filtration on Sephadex G-25. The active portion (a range of 1.9–2.7 × hold-up volume) was lyophilized and then applied on an ion-exchange HPLC column (4.0 × 300 mm, TSK IEX530 CM SIL, Toyo Soda, Japan). The column was eluted with 10% $CH_3CN$, using a linear gradient elution of ammonium formate (pH 4.7) from 10 mM to 0.5 M. The active portion (Fig. 1, black bar) was finally purified by reverse-phase HPLC on a TSK LS410 $C_{18}$ column (4.0 × 250 mm, Toyo Soda). The column was eluted with 0.1% trifluoroacetic acid by using a linear gradient elution of $CH_3CN$ from 10% to 60% (Fig. 2). The column was monitored by measuring absorbance at 210 nm and 280 nm.

**Enzymatic Digestion of Chicken GnRH-II.** Natural chicken GnRH-II (1 μg) was digested with 2 μg of α-chymotrypsin (Sigma) in 5 μl of 0.2 M N-ethylmorpholin acetate buffer (pH 8.0) at 37°C for 4 hr. The digest was then applied to a HPLC column (Chemcosorb $C_{18}$, 3 μm) and eluted with 0.1% trifluoroacetic acid, using a linear gradient elution of $CH_3CN$ from 0% to 60% at a flow rate of 1.0 ml/min. Thermolytic digestion was also performed, and the resulting digest was submitted to HPLC under the conditions described above. Chymotryptic (designated ChT-I to ChT-IV) and thermolytic (designated Th-I and Th-II) peptides derived from the starting peptide were isolated on the HPLC system and were used for structural analysis.

**Amino Acid Analysis.** Peptides (0.3–1.0 nmol) were hydro-



FIG. 2. Final purification of chicken GnRH-II on a reverse-phase HPLC column. Column: 4.0 × 250 mm, TSK-LS410 C-18; solvent system: a linear gradient (80 min; flow rate, 2.0 ml/min) from 0.1% trifluoroacetic acid/$CH_3CN$, 90:10 (vol/vol), to 0.1% trifluoroacetic acid/$CH_3CN$, 40:60 (vol/vol). The active portion is marked with a black bar.

lyzed in 6 M HCl containing 0.1% 2-mercaptoethanol at 110°C for 20 hr. The hydrolysates were analyzed on a Hitachi 835 amino acid analyzer.

**Terminal Analyses.** All peptides (100–300 pmol) were used in $NH_2$-terminal analysis by dansylation as described (8). About 200 pmol of COOH-terminal peptide derived from thermolytic cleavage (Th-II) was digested with postproline-cleaving enzyme (Seikagaku Kogyo, Japan), and liberated COOH-terminal Gly-$NH_2$ was identified as dansyl-Gly-$NH_2$ by the method of Tatemoto and Mutt (9).

$NH_2$-terminal analysis was also carried out by subtractive Edman degradation with a sensitive amino acid analyzing system in which amino acids were prelabeled with o-phthalaldehyde in the presence of 2-mercaptoethanol (10).

**Synthesis of Chicken GnRH-II.** Chicken GnRH-II with the structure of pGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-$NH_2$ was synthesized by solid-phase techniques conducted on a p-methylbenzhydrylamine resin for the purpose of structural identification. The crude peptide preparation obtained after HF treatment was incubated in 1% piperidine solution (pH 10.0) at 37°C for 2 hr to remove formyl groups from the two tryptophan residues and then purified on a reverse-phase HPLC column. Purity of the peptide was verified by HPLC. Amino acid analyses and sequencing of the peptide confirmed correct synthesis.

**Comparison of Natural and Synthetic Chicken GnRH-II on HPLC.** Synthetic chicken GnRH-II was also digested with chymotrypsin and thermolysin, and the digests were analyzed by the HPLC system under exactly the same conditions as applied for the natural preparation.

## RESULTS

**Isolation of Chicken GnRH-II.** Chicken GnRH-II was well separated from chicken LHRH, which had been isolated and characterized in our laboratory (3, 4), at an early stage of purification. On a SP-Sephadex G-25 column, chicken GnRH-II was eluted with 2 M pyridine acetate buffer (pH 5.0), while chicken LHRH was eluted with 2 M pyridine. Chicken GnRH-II emerged at a fairly retarded position, compared with authentic chicken LHRH and mammalian LHRH on a cation-exchange HPLC column (Fig. 1), indicating its strong hydrophobic interaction with the column.

The active portion was finally purified on a reverse-phase HPLC column (Fig. 2). The pure peptide was obtained in a
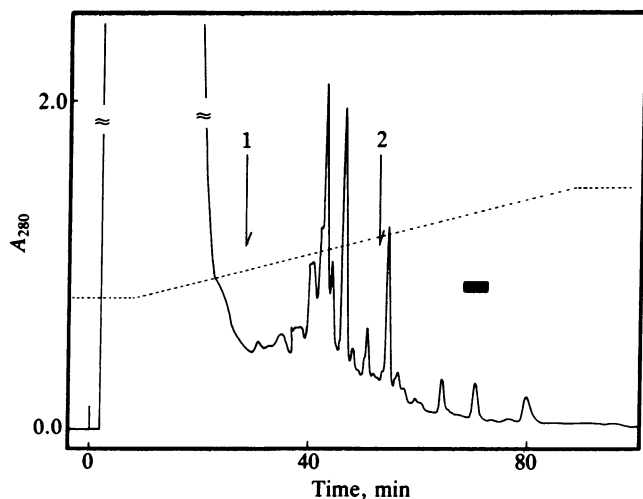


FIG. 1. Cation-exchange HPLC of a small peptide portion obtained after Sephadex G-25 gel filtration of the chicken hypothalamic extract, followed by batchwise treatment with SP-Sephadex C-25 (free form). Column: 4.0 × 300 mm, TSK IEX530 CM SIL (cation exchange); solvent system: a linear gradient (80 min; flow rate, 1.0 ml/min) from 10 mM ammonium formate, pH 4.7/$CH_3CN$, 90:10 (vol/vol), to 0.5 M ammonium formate, pH 4.7/$CH_3CN$, 90:10 (vol/vol). The active portion is marked with a black bar. Arrows indicate the elution positions of chicken LHRH (1) and mammalian LHRH (2).
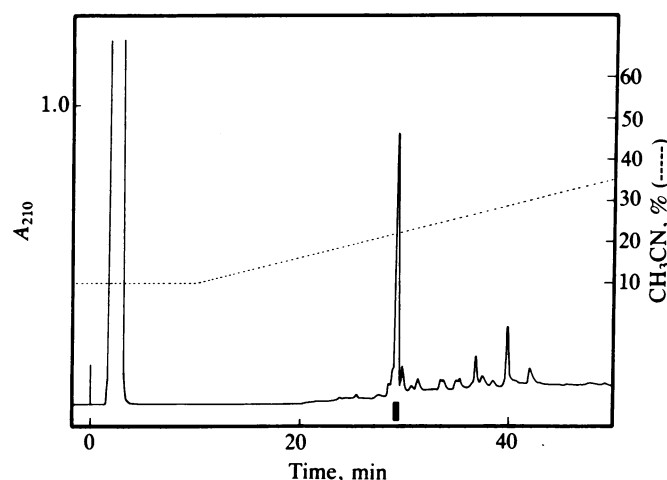
Table 1. Amino acid compositions of peptides derived from chicken GnRH-II

| Amino acid | GnRH-II | ChT-I | ChT-II | ChT-III | ChT-IV | Th-I | Th-II |
|---|---|---|---|---|---|---|---|
| Ser | 1.25 (1) | — | 1.00 (1) | — | 1.20 (1) | — | — |
| Glx | 1.00 (1) | 1.00 (1) | — | — | — | 1.00 (1) | — |
| Pro | 1.01 (1) | — | — | 1.00 (1) | 0.75 (1) | — | 1.12 (1) |
| Gly | 2.00 (2) | — | 1.05 (1) | 1.22 (1) | 2.07 (2) | — | 2.00 (2) |
| Tyr | 0.96 (1) | — | — | 0.83 (1) | 1.00 (1) | — | 1.00 (1) |
| His | 1.56 (2) | 0.83 (1) | 0.85 (1) | — | 0.97 (1) | 0.92 (1) | — |
| Trp* | 0.54 (2) | 0.20 (1) | + (1) | — | 0.67 (1) | 0.42 (1) | 0.69 (1) |
| No. of residues | 10 | 3 | 4 | 3 | 7 | 3 | 5 |

ChT, chymotrypsin; Th, thermolysin.
*Tryptophan residues were partly destroyed under the hydrolytic conditions described in the text. The integral numbers of tryptophan residues in parentheses were also deduced by simultaneously monitoring the ratios of absorbance of the peptides at 210 nm and 280 nm.

yield of 7 μg (based upon its amino acid analysis) starting from 10,000 fragments of chicken hypothalami.

When rat anterior pituitary cells were used for the bioassay, 60% of the total activity in chicken hypothalamic extract was due to GnRH-II.

**Structure of Chicken GnRH-II.** The amino acid analysis (Table 1) indicates that chicken GnRH-II is a decapeptide.

HPLC patterns of thermolytic and chymotryptic digests of natural chicken GnRH-II are shown in Fig. 3 *Left Middle* and *Left Bottom*, respectively. Chymotryptic digestion resulted in the formation of four peptide fragments (ChT-I to ChT-IV), and thermolytic digestion gave two fragments (Th-



**FIG. 3.** Reverse-phase HPLC of natural (*Top Left*) and synthetic (*Top Right*) chicken GnRH-II, thermolytic fragments of natural (*Middle Left*) and synthetic (*Middle Right*) GnRH-II, and chymotryptic fragments of natural (*Bottom Left*) and synthetic (*Bottom Right*) decapeptide. Sample loaded: 50 ng of natural and synthetic GnRH-II (*Top Left* and *Right*, respectively), thermolytic digest of natural (2 μg) and synthetic GnRH-II (1 μg) (*Middle Left* and *Right*, respectively), and chymotryptic digest of natural (1 μg) and synthetic GnRH-II (1 μg) (*Bottom Left* and *Right*, respectively). Column: Chemcosorb C₁₈ 3 μm (4.6 × 75 mm); elution: a linear gradient of 0.1% trifluoroacetic acid containing CH₃CH, from 0% to 60%; flow rate: 1 ml/min. Arrows indicate elution positions of isolated peptide fragments. See Table 1 and Fig. 4.

I and Th-II) along with a small amount of unreacted starting peptide (N). Amino acid compositions of these peptides are listed in Table 1.

The complete structure of chicken GnRH-II has been determined on the basis of the amino acid compositions of these peptide fragments, along with the results of their terminal analyses (Fig. 4). It is notable that the linkages of Trp³-Ser⁴ and His⁵-Gly⁶ were predominantly cleaved with thermolysin in this case. Because of the hydrophobic nature of this peptide, the solubility of the peptide in the digestion buffer was limited, and a large amount of proteolytic enzymes (2 μg per 5 μl) were required to cleave it quantitatively.

Further structural confirmation is provided by chromatographic comparison of the synthetic chicken GnRH-II with the natural preparation, as shown in Fig. 3 *Left Top* and *Right Top*, in which the two peptides are eluted at exactly the same position. In addition, it is obvious that synthetic chicken GnRH-II undergoes enzymatic cleavage (Fig. 3 *Right Middle* and *Bottom*) in a manner identical with the natural preparation (Fig. 3 *Left Middle* and *Bottom*).

Consequently, the identity of natural chicken GnRH-II with the synthetic decapeptide has definitely been confirmed. Furthermore, the gonadotropin-releasing activity of the synthetic chicken GnRH-II was also found to show a good agreement with that of the natural preparation (Fig. 5). When rat anterior pituitary cells were used for the bioassay, the LH-releasing potency of chicken GnRH-II was about 32% of that of mammalian LHRH, and it was about 8 times more potent than chicken LHRH. This peptide also can stimulate the secretion of FSH as well as LH *in vitro*, and the relative LH- and FSH-releasing potency of chicken GnRH-II is summarized in Table 2 together with those of mammalian and chicken LHRH.

## DISCUSSION

A naturally occurring GnRH distinct from mammalian LHRH was first isolated from chicken hypothalamus and characterized as [Gln⁸]LHRH by our group (3, 4) and King and Millar (5–7). Quite recently Sherwood *et al.* (11) reported the chemical structure of salmon GnRH.
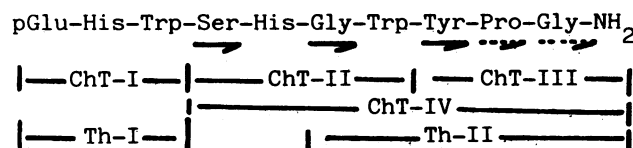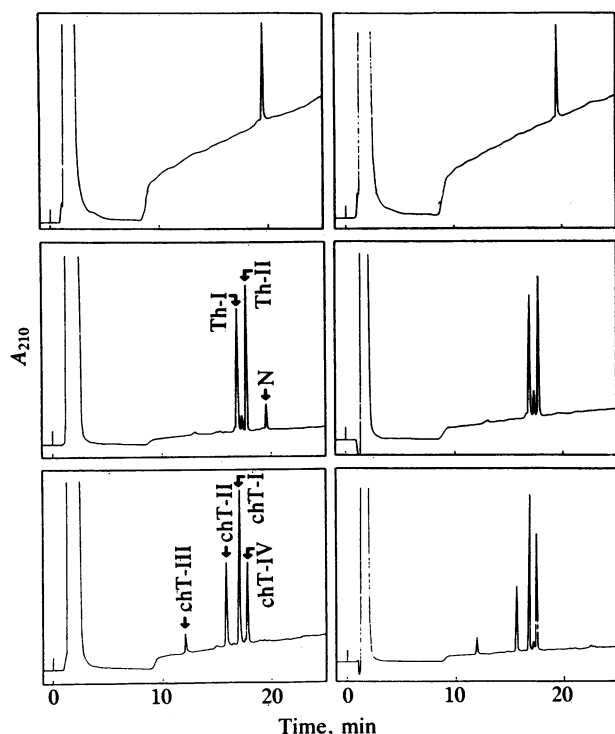


**FIG. 4.** Structure of chicken GnRH-II. ChT, chymotryptic peptides; Th, thermolytic peptides; →, determined by Dansyl procedure; ----→, determined by postproline-cleaving enzyme treatment followed by dansylation.

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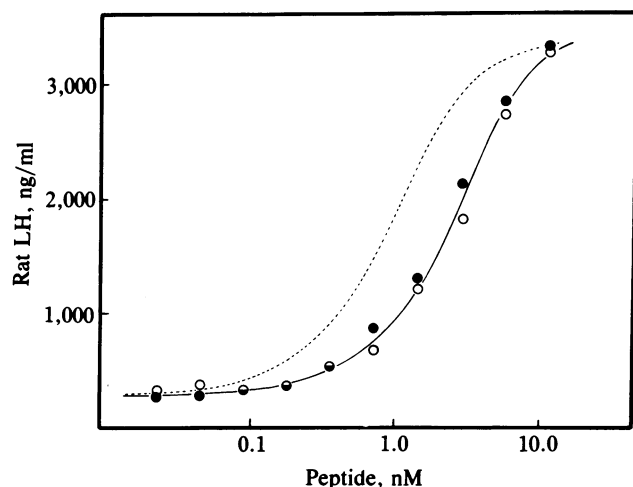*Proc. Natl. Acad. Sci. USA 81 (1984)*     3877



FIG. 5. Effect of natural (●) and synthetic (○) chicken GnRH-II on the secretion of LH by monolayer culture of rat pituitary cells. Day 4 cultures were incubated with natural or synthetic chicken GnRH-II in 0.2 ml of medium for 24 hr at 37°C. The data are shown as the means of triplicate determinations. -----, Dose–response curve of mammalian LHRH.

Naturally occurring GnRHs characterized so far are shown in Fig. 6, including chicken GnRH-II.

Gonadotropin secretion in mammalian species has been believed to be regulated by the sole GnRH mammalian LHRH. However, we have demonstrated that the secretion of FSH under certain physiological and experimental conditions is not controlled by mammalian LHRH in rats (2). It might be possible that another gonadotropin-releasing factor distinct from mammalian LHRH is present and functions in mammalian hypothalamus to control the physiological secretion of LH and FSH. However, no structural evidence has been reported indicating that two distinct GnRHs coexist and function in the hypothalamus of mammalian species as well as in the brain of any other submammalian vertebrates.

The present report demonstrates that two distinct GnRHs exist in one species, suggesting that gonadotropin secretion is probably regulated by a dual mechanism. We also have observed that two or more than two gonadotropin-releasing substances chromatographically distinguished from each other coexist in frog and rat brains as well as in porcine hypothalamus (unpublished data). It seems likely that a multiple system of GnRHs exists not only in chicken hypothalamus but also in the hypothalamus of other mammalian and submammalian vertebrates.

The systematic study of structure–activity relationship in the LHRH molecule using synthetic analogs of LHRH (12) has revealed that the major structural moieties important for the bioactivity are the $NH_2$-terminal sequence pGlu-His-Trp, the 6 position (Gly), and the COOH-terminal moiety of Pro-Gly-$NH_2$. As shown in Fig. 6, the above structure–activity relationship is also preserved in the case of the natural GnRHs—that is, the length of the chain, the $NH_2$ and COOH termini, and amino acids in positions 1–4, 6, 9, and 10 are stable throughout the four GnRHs.

It is notable in Fig. 6 that three of the four GnRHs, mam-

Table 2. Relative LH- and FSH-releasing potencies of the peptides acting on rat pituitary cells

| Peptide | LH | FSH |
| --- | --- | --- |
| Mammalian LHRH | 1.00 | 1.00 |
| Chicken GnRH-II | 0.32 | 0.41 |
| Chicken LHRH | 0.034 | 0.032 |

The potency of mammalian LHRH is taken as 1.00.

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chicken  GnRH-II;  pGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH₂
chicken  LH-RH;    pGlu-His-Trp-Ser-Tyr-Gly-Leu-Gln-Pro-Gly-NH₂  (1-5)
mammalian LH-RH;   pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH₂   (6)
teleost  Gn-RH;    pGlu-His-Trp-Ser-Tyr-Gly-Trp-Leu-Pro-Gly-NH₂   (9)
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FIG. 6. Structures of naturally occurring GnRHs.

malian LHRH, chicken LHRH, and salmon GnRH are convertible from each other by single-base changes in genetic codons. The Gln residue in chicken LHRH can be converted to an Arg or Leu residue of mammalian LHRH or salmon GnRH by a single base nucleotide substitution, respectively. The Trp residue at position 7 of salmon GnRH is also convertible to the Leu residue of mammalian or chicken LHRH in that way. On the other hand, chicken GnRH-II is unable to be converted to the other three GnRHs by point mutation. The Tyr residue at position 8 of chicken GnRH-II is not convertible to the respective amino acids of the other three GnRHs by a single-base nucleotide substitution. These observations suggest that the evolution of chicken GnRH-II has occurred at an early stage of development compared with those of the other three GnRHs.

The structural characteristics of chicken GnRH-II also has been shown by the fact that thermolysin treatment gave rise to only two major peptides, w hereas no less than five fragments were obtained after cleavage of chicken LHRH by this enzyme (4). The hydrophobic or adsorptive nature of this peptide might be one of the reasons for such a phenomenon because the solubility of the peptide in the digestion buffer was limited and a large amount of proteolytic enzymes were required to cleave it quantitatively.

It has been established that there exist multihormone precursors, such as the corticotropin-$\beta$-lipotropin precursor (13), the adrenal preproenkephalin (14), and the $\beta$-neo-endorphin-dynorphin precursor (15), from which hormones or opioid peptides are produced by their specific processing enzymes. It is also possible that the two GnRHs, chicken LHRH and GnRH-II, are involved in a common precursor molecule.

Two gonadotropins, FSH and LH, are present not only in mammalian but also in submammalian species, such as birds, reptiles, and amphibians (16). The regulation of the two gonadotropins in these species is still unclear.

The ratio of LH- and FSH-releasing activity of chicken GnRH-II is similar to that of mammalian LHRH, when rat anterior pituitary cells were used for the bioassay. However, the LH- and FSH-releasing potency of chicken GnRH-II may be different when chicken pituitary cells and chicken LH and FSH radioimmunoassay systems are used.

We speculate that two GnRHs in chicken hypothalamus, chicken LHRH and chicken GnRH-II, may serve as LHRH and FSH-releasing hormone, respectively, in avian species.

1.  Matsuo, H., Baba, Y., Nair, R. M. G., Arimura, A. & Schally, A. V. (1971) *Biochem. Biophys. Res. Commun.* 43, 1334–1339.
2.  Hasegawa, Y., Miyamoto, K., Yazaki, C. & Igarashi, M. (1981) *Endocrinology* 109, 130–135.
3.  Miyamoto, K., Hasegawa, Y., Minegishi, T., Nomura, M., Takahashi, Y., Igarashi, M., Kangawa, K. & Matsuo, H. (1982) *Biochem. Biophys. Res. Commun.* 107, 820–827.
4.  Miyamoto, K., Hasegawa, Y., Igarashi, M., Chino, N., Sakakibara, S., Kangawa, K. & Matsuo, H. (1983) *Life Sci.* 32, 1341–1347.
5.  King, J. A. & Millar, R. P. (1982) *S. Afr. J. Sci.* 78, 124–125.
6.  King, J. A. & Millar, R. P. (1982) *J. Biol. Chem.* 257, 10722–10728.
7.  King, J. A. & Millar, R. P. (1982) *J. Biol. Chem.* 257, 10729–10732.
8.  Lai, C. Y. (1977) *Methods Enzymol.* 47, 236–243.
9.  Tatemoto, K. & Mutt, V. (1978) *Proc. Natl. Acad. Sci. USA* 75, 4115–4119.

10. Méndez, E. (1982) *Anal. Biochem.* **127,** 55–60.
11. Sherwood, N., Eiden, L., Brownstein, M., Spiess, J., Rivier, J. & Vale, W. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 2794–2798.
12. Sandow, J., Konig, W., Geiger, R., Uhmann, R. & Rechenberg, W. (1978) in *Control of Ovulation*, eds. Crighton, D. B., Foxcroft, G. R., Haynes, N. B. & Lamming, G. E. (Butterworth, London), pp. 49–70.
13. Nakanishi, S., Inoue, A., Kita, T., Nakamura, M., Chang, A. C. Y., Cohen, S. N. & Numa, S. (1979) *Nature (London)* **278,** 423–427.
14. Noda, M., Furutani, Y., Takahashi, H., Toyosato, M., Hirose, T., Inayama, S., Nakanishi, S. & Numa, S. (1982) *Nature (London)* **295,** 202–206.
15. Kakidani, H., Furutani, Y., Takahashi, H., Noda, M., Morimoto, Y., Hirose, T., Asai, M., Inayama, S., Nakanishi, S. & Numa, S. (1982) *Nature (London)* **298,** 245–249.
16. Ball, J. N. (1981) *Gen. Comp. Endocrinol.* **44,** 135–170.